Phylogenetically Informative Length Polymorphism and Sequence Variability in Mitochondrial DNA of Australian Songbirds (*Pomatostomus*)

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ABSTRACT

A combination of restriction analysis and direct sequencing via the polymerase chain reaction (PCR) was used to build trees relating mitochondrial DNAs (mtDNAs) from 50 individuals belonging to five species of Australian babblers (*Pomatostomus*). The trees served as a quantitative framework for analyzing the direction and tempo of evolution of an intraspecific length polymorphism from a third mitochondrial ancestor. The length polymorphism lies between the cytochrome *b* and 12S rRNA (srRNA) genes. Screening of mtDNAs within and between the five species with restriction enzymes showed that *Pomatosomus temporalis* was polymorphic for two smaller size classes (M and S) that are completely segregated geographically, whereas mtDNAs from the other four species were exclusively of a third, larger size (L). Inter- and intraspecific phylogenetic trees relating mtDNAs based on restriction maps, cytochrome *b* sequences obtained via PCR, and the two data sets combined were compared to one another statistically and were broadly similar except for the phylogenetic position of *Pomatosomus halli*. Both sets of phylogenies imply that only two deletion events can account for the observed intraspecific distribution of the three length types. High levels of base-substitutional divergence were detected within and between northern and southern lineages of *P. temporalis*, which implies a low level of gene flow between northern and southern regions as well as a low rate of length mutation. These conclusions were confirmed by applying coalescent theory to the statistical framework provided by the phylogenetic analyses.

RESTRICTION analysis and direct sequencing via the polymerase chain reaction (PCR) offer geneticists two ways to answer questions about relationships of individuals within and among species and about structural evolution of mitochondrial DNA (WILSON *et al.* 1985; AVISE *et al.* 1987; MORITZ, DOWLING and BROWN 1987; WRISCHNIK *et al.* 1987; VIGILANT, STONEKING and WILSON 1988; HARRISON 1989; KOCHER *et al.* 1989; THOMAS *et al.* 1989; THOMAS, MAA and WILSON 1989; VIGILANT *et al.* 1989; THOMAS *et al.* 1990). mtDNA variation has been characterized before in a few birds (MACK *et al.* 1986; SHIELDS and WILSON 1987b; OVENDEN, MACKINLAY and CROZIER 1987; BALL *et al.* 1988). With respect to patterns of mtDNA variation, bird species display a wide variety of population structures ranging from near panmixia (BALL *et al.* 1988) to discrete geographic and subspecific differentiation (SHIELDS and WILSON 1987b). In addition to abundant restriction site polymorphisms, intraspecific variation in total size of mtDNAs has been reported for two avian species (AVISE and ZINK 1988). mtDNA length differences have also been detected in many other animal species from nematodes to humans (reviewed in MORITZ, DOWLING and BROWN 1987). In those cases where length variation was known to accompany differences detected with restriction enzymes, more often than not the length differences show little geographic or phylogenetic concordance with cleavage site differences (CANN and WILSON 1983; HARRISON, RAND and WHEELER 1985; BENTZEN, LEGGETT and BROWN 1988). Exceptions to this generalization include length mutations in rat mtDNA (HAYASHI *et al.* 1981), small (1-bp) deletions in the small rRNA gene in primates (HIXSON and BROWN 1986), and an intergenic length mutation in human mtDNA (WRISCHNIK *et al.* 1987), all of which proved concordant with a specific phylogenetic hypothesis derived from restriction maps or sequences. WRISCHNIK *et al.* (1987) amplified and sequenced regions in multiple human mtDNAs suspected by restriction analysis to exhibit length polymorphisms. These sequences were used to refine previously published gene trees based solely on restriction analysis and to help identify a mtDNA length mutation that may prove reliable as a population marker.

Here we analyze mitochondrial DNA evolution in babblers belonging to the Australo-Papuan songbird genus *Pomatostomus* using a combination of restriction enzyme analysis and direct sequencing via PCR. These babblers are best known to behavioral ecologists for their conspicuous sociality and family group structure

(BOEHM 1974; BROWN 1987 and references therein). All five species are cooperative breeders in that several, often related birds will assist a mated pair with nest building and raising of the brood. The genus is also of interest phylogenetically because of the recent proposal based on DNA hybridization that they are related more closely to other Australian songbirds rather than to Palearctic babblers (SIBLEY and AHLQUIST 1985; SIBLEY, AHLQUIST and MONROE 1988). This paper characterizes and analyzes the inter- and intraspecific distribution of a trio of discrete mtDNA length variants. We used PCR to amplify and directly sequence a portion of the cytochrome *b* gene of a subset of these mtDNAs in order to assess the concordance of phylogenetic trees based on analysis of restriction sites and DNA sequences from the same genomes. These analyses and combined data sets allow us to answer two questions concerning the evolution of mtDNA in *Pomatostomus* and of relevance to the study of mtDNA length variation generally: (1) what is the phylogeny of the length variants within and between species and what does this phylogeny suggest about the rate and pattern of genome size evolution in *Pomatostomus*? and (2) what does the pattern of base substitution within *Pomatostomus temporalis* imply about population structure and dynamics and their influence on the geographic distribution of mtDNA length variants in this species?

MATERIALS AND METHODS

Field sampling and collecting localities: Babbler populations in Australia were sampled at eight localities (A–H) from February to May 1987 (Table 1). All babbler species are structured into sedentary, territorial, spatially distinct family groups consisting of a breeding pair and several helpers (BROWN 1987). These groups are easily delimited and identifiable in the field. Each individual collected was assigned to a numbered family group based on the spatial locations of family groups within each locality. Livers and hearts were dissected and placed in nunc tubes in liquid nitrogen (−196°) within 2 hr of collection. The samples of *P. temporalis* include representatives of the two main subspecies, *temporalis* in the south and east and *rubeculus* in the north (HALL 1974). The two samples of *Pomatostomus isidori* (Table 1) were collected in Papua New Guinea by L. CHRISTIDIS and R. SCHODDE. Tissue samples were flown on dry ice from Australia and stored at Berkeley.

mtDNA isolation, restriction analysis and mapping: mtDNA was highly purified by CsCl gradient centrifugation essentially as described in BROWN (1980) and SHIELDS and WILSON (1987a), except that it was often necessary to spin the initial lysate of cells three or four times at 5000 rpm to pellet nuclei and cellular debris more completely. A total of 74 mtDNAs from 51 family groups among the five species were isolated and stored at −20° for length typing, restriction mapping or enzymatic amplification. Restriction digestion was conducted for 1–3 hr at 37° in reactions consisting of 8–16 μl purified mtDNA (1 μg/ml), 2 μl of the appropriate buffer, 1 μl bovine serum albumin (1 μg/ml), 5–10 units restriction enzyme, and doubly distilled water for a total of 20 μl. Restriction fragments were end-labeled by adding 1 μl of large fragment Klenow DNA polymerase (New England Biolabs), 1 μl each of the appropriate [^{32}P]- or [^{35}S] dNTPs, 2 μl buffer and 16 μl doubly distilled water. All 74 mtDNAs were screened for length type using *Sac*II/*Bam*HI double digests; in profiles of *Pomatostomus halli* mtDNA or those *P. temporalis* mtDNAs in which none of the diagnostic fragments was present, digestion with other enzyme combinations and electrophoresis in 0.7–1.0% agarose gels permitted length classification. Fifty of the 74 mtDNAs were digested with six more enzymes recognizing six bases: *Hin*dIII, *Sal*I, *Xba*I, *Cla*I, *Bgl*II and *Eco*RI. Restriction digests were analyzed by electrophoresis in 0.7–1.2% agarose and 40-cm 6% polyacrylamide gels. Among the five species, six mtDNA types were chosen for mapping of sites produced by the eight enzymes, using double digestion with 10–15 combinations of enzymes. For the remaining mtDNA types, site differences were inferred directly from fragment pattern comparisons if profiles were similar to one of the maps, or from additional double digests.

Genomic DNA isolation and Southern blotting: Each pellet (1–1.5 g) from the initial centrifugations used to isolate mtDNA was transferred to a 50-ml sterile capped plastic tube and suspended in 5 ml of STE buffer (50 mM sodium chloride, 5 mM disodium ethylenediamine tetraace-

TABLE 1

Collecting localities and number of babbler mtDNAs studied

Place of collection[a]	No. of mtDNAs (from *n* family groups)[b]	
	Screened for length polymorphism	Mapped
P. temporalis		
A. Blowclear West State Forest, NSW	8 (7)	7 (7)
B. Musheroo property, Cobar, NSW	9 (4)	9 (4)
C. Goonamurra property, Eulo, Qld.	1 (1)	1 (1)
D. Cloncurry, Qld.	11 (5)	10 (5)
E. near Woolner Station, NT	9 (6)	8 (6)
A–E	38 (23)	35 (23)
P. superciliosus		
A. Blowclear, as above	8 (7)	1 (1)
F. Blanchtown, SA	3 (2)	1 (1)
G. Kimba, SA	3 (3)	1 (1)
A, F, G	14 (12)	3 (3)
P. ruficeps		
B. Musheroo, as above	5 (3)	2 (2)
C. Goonamurra, as above	1 (1)	1 (1)
F. Blanchtown, as above	7 (6)	3 (3)
H. Cunnamulla, Qld.	4 (2)	2 (2)
B, C, F, H	17 (12)	8 (8)
P. isidori		
I. near Port Moresby, PNG	3 (2)	2 (2)
P. halli		
C. Goonamurra, as above	1 (1)	1 (1)
J. 96 km west of Charleville, Qld.	1 (1)	1 (1)
C, J	2 (2)	2 (2)
All places, all species	74 (51)	50 (38)

[a] For *P. temporalis* populations, babbler family groups within each locality were on average about 0.3 km apart. The average "area" of each locality was five square kilometers. Exact localities can be obtained from S. V. EDWARDS. Abbreviations for regions in Australia are NSW, New South Wales; Qld., Queensland; NT, Northern Territory; SA, South Australia. PNG is Papua New Guinea.

[b] See MATERIALS AND METHODS.

tate, 10 mM Tris, pH 8.0), 1.4 ml of 10% sodium dodecyl sulfate, and 1.5 mg of Proteinase K (Sigma). The solutions were incubated at 25–30° overnight with slight agitation; cesium chloride equivalent to 1.25 times the weight of the solution was then added and dissolved. The final solutions were transferred to 13-ml sealable ultracentrifuge tubes (Beckman), 4 μl of ethidium bromide (2 mg/ml) added, and the tubes spun at 45,000 rpm for 24 hr. The band of genomic DNA was visualized under UV light and extracted with an 18-gauge syringe. The DNA was cleaned of ethidium bromide and protein contaminants with several extractions with *n*-butanol and then precipitated with cold ethanol and stored in 10 mM Tris, 1 mM ethylene diamine tetraacetate at −20°.

Genomic DNA (10 μg) was electrophoresed in 0.7–1.0% agarose gels and Southern blotting (SOUTHERN 1975) of nuclear DNA was performed as described by KAN and DOZY (1978). Probes specific to mitochondrial genes were produced by enzymatic amplification of specific segments of mtDNA using purified mtDNAs as templates (see below). In each case the amplified product was extracted from a 2% low melting point agarose preparative gel with a razor blade, kept at 37° to prevent congealing, and ^{32}P-labeled with random oligonucleotide primers (FEINBERG and VOGELSTEIN 1983).

Polymerase chain reaction and direct sequencing: For use as probes on Southern blots, portions of the mitochondrial srRNA (386 bp) and cytochrome *b* (307 bp) genes were amplified using, respectively, primer pairs L1091-H1478 and L14841-H15149 (KOCHER *et al.* 1989). Single-stranded templates for sequencing were produced by the unbalanced primer method (GYLLENSTEN and ERLICH 1988) from double-stranded templates and sequenced using the limiting primer in the second PCR. A fifth primer, H15915 (5′-AACTGCAGTCATCTCCGGTTTACAAGAC-3′), was used with L14841 to amplify a 1073-bp segment for use as a second cytochrome *b* probe. One or two individuals representing each mtDNA type as defined by restriction analysis were chosen for amplification of the 307-bp cytochrome *b* segment and sequenced as described (KOCHER *et al.* 1989).

Phylogenetic and statistical analyses: Each distinct restriction digestion pattern produced was given a letter designation. The matrix of restriction sites was analyzed with PAUP version 3.0b (SWOFFORD 1989), using Wagner parsimony, in which the interconversions between any two character states are equally likely, and Dollo parsimony (DEBRY and SLADE 1985), in which character (restriction site) gains are minimized. Percent sequence divergence was estimated from restriction maps by the method of WILSON *et al.* (1989). Cytochrome *b* sequences were aligned by eye using the program ESEE (CABOT and BECKENBACH 1989) and analyzed phylogenetically using PAUP (SWOFFORD 1989). In each analysis, rooting was performed using an appropriate outgroup. Confidence in particular branches was assessed using the bootstrap procedure (FELSENSTEIN 1985). Bootstrapping was performed on matrices in which only the informative sites were included, and in the intraspecific (*P. temporalis*) analyses of restriction side data, the maximum number of trees stored per bootstrap replicate was set at 100. Statistical superiority of particular trees over competing hypotheses was assessed using the winning-sites method (PRAGER and WILSON 1988). For the interspecific analyses, an appropriate subset of the *P. temporalis* mtDNAs was used, whereas in the combined set of restriction and cytochrome *b* sequence data, all individuals were included.

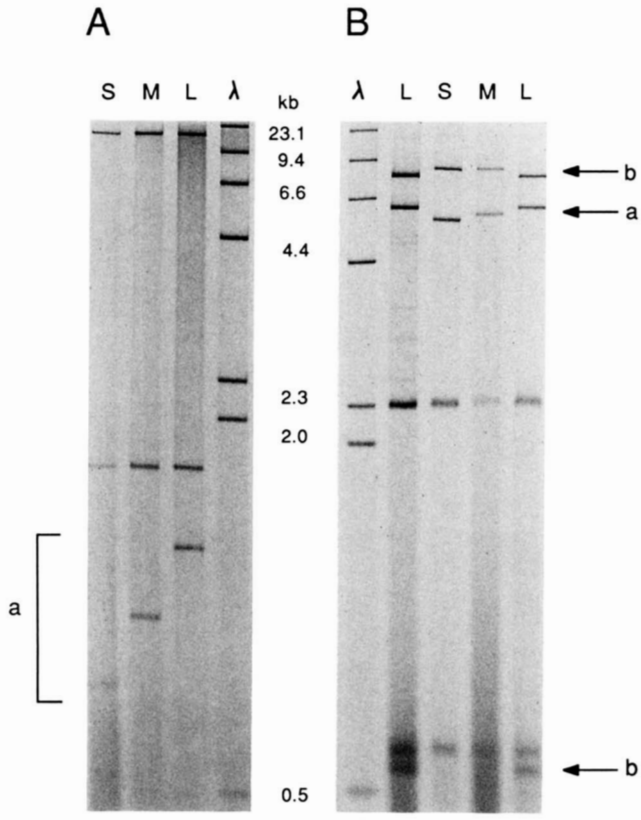

FIGURE 1.—Three classes of length variants shown by gel electrophoresis of restriction digests of purified babbler mtDNA. Lanes contain mtDNAs designated small (S), medium (M), and large (L) size classes of mtDNA that were cut in double digests and end-labeled with ^{35}S as described in MATERIALS AND METHODS. A, *Sac*II/*Bam*HI double digests. All three lanes with mtDNA have the genotype pattern "AA" for these enzymes (Table 2). The bracket labeled *a* indicates fragments bearing length mutations. B, *Hin*dIII/*Bam*HI double digests. The two L lanes show fragment pattern "BA," and the S and M lanes both show pattern "AA" (Table 2). The arrow marked *a* indicates fragments bearing length mutations, whereas the arrows marked *b* indicate fragment size variation due to restriction site variation (note 0.6-kb fragments in L lanes). Lanes marked λ contain bacteriophage lambda DNA digested with *Hin*dIII. kb = kilobase pairs.

RESULTS

mtDNA length variation: Electrophoretic analysis of initial double digests involving *Hin*dIII, *Bam*HI, and *Sac*II made it evident that discrete length differences in addition to restriction site changes might be causing variation in digestion profiles both within and between species (Figure 1). *Sac*II/*Bam*HI digestion profiles corresponding to the pattern "AA" (Table 2) exhibited three fragments, whose mobilities corresponded to either 0.9, 1.1, or 1.4 kb (Figure 1A). Digestion profiles produced by enzymes that cut the mtDNAs more frequently than those in Figure 1A were nonetheless consistent with the *Sac*II/*Bam*HI double digests in that they possessed one fragment that varied in length by increments of 200 and 300 bp between individuals (Figure 1B). Consistent with the hypothesis of length variation was the observation

TABLE 2

Sizes of fragments in digestion profiles showing discrete length variation in babbler mtDNAs

	Fragment size in kilobase pairs					
	temporalis[a]					
Enzyme combination	S	M	*superciliosus*	*ruficeps*	*isidori*	*halli*
*Sac*II/*Bam*HI	(AA)	(AA)	(BA)	(BA)	(AD)	(AE)
	14+	14+	10+	10+	5.7	12−
	1.7	1.7	4.0	4.0	4.3	1.9
	0.9*	1.1*	1.7	1.7	2.6	1.7
			1.4*	1.4*	1.7	1.0
					1.6	0.6
					1.4*	0.4
*Hin*dIII/ *Bam*HI	(AA)	(AA)	(AA)	(BA)		
	8.2	8.2	8.2	7.6	ND	ND
	5.6*	5.8*	6.1*	6.1*		
	2.3	2.3	2.3	2.3		
	0.7	0.7	0.7	0.7		
				0.6		
*Cla*I/*Bam*HI	(AA)	(AA)	(CA)	(AA)	(ED)	
	8.0	8.0	8.0	8.0	5.9	ND
	7.7	7.7	5.2	7.7	4.1	
	1.1*	1.3*	2.5	1.6*	2.85	
			1.6*		2.8	
					1.65	
*Xba*I/*Bam*HI	(AA)	(CA)	(BA)	(FA)	(ED)	
	10−*	10*	7.0	12−	5.3	ND
	6.9	6.4	5.5	5.5	3.85	
		0.5	3.7		3.75	
			1.1		2.7	
					1.7	
*Sal*I	(B)	(D)	(F)	(E)	(G)	(D)
	9.6*	9.8*	15+	9.1	17+	10+*
	7.2	5.6	1.7	6.8		5.6
		1.4		1.4		1.4
		0.25				0.25

Capital letters in parentheses refer to digestion patterns for the individual enzymes (see Table 6, APPENDIX). Asterisks denote fragments bearing length mutations. Plus or minus signs next to large fragments (≥10 kbp) indicate an approximate size deviation of the fragment from the size given to the nearest kbp. ND, not done.

[a] S and M indicate small and medium genome size classes.

that after different single and double digestions, specific mtDNA fragments varied discretely and predictably in accordance with each mtDNA's *Sac*II/*Bam*HI profile (Table 2). These fragment differences persisted upon exhaustive digestion and were present in multiple double and single digestion profiles (Table 2).

Distribution of mtDNA length variation among babblers: A total of 74 mtDNAs from 51 babbler family groups were screened for length variation with *Sac*II and *Bam*HI (Table 1), and were designated small (S), medium (M), or large (L) depending on the presence of the 0.9-, 1.1- or 1.4-kb fragment, respectively. *Sal*I digestion profiles of *P. halli* mtDNA suggested it belonged to the large size class (Table 2), and electrophoresis of *P. temporalis* mtDNA types 11 and 13 (Table 6 in the APPENDIX) in lower density gels (see MATERIALS AND METHODS) indicated that both were of small size (not shown). Heteroplasmy, or the presence of multiple size classes of mtDNA within individual samples, was not detected in any samples.

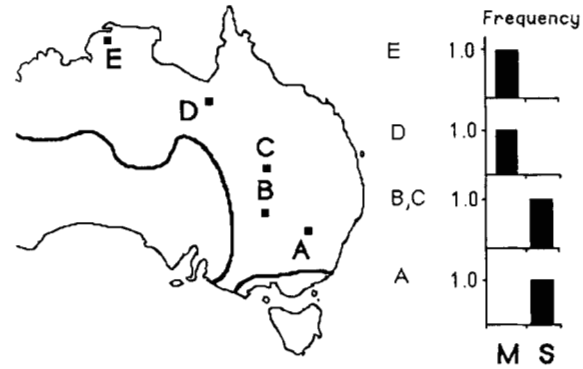

FIGURE 2.—Geographic distribution and frequencies of small (S) and medium (M) mtDNA length variants in five populations of *P. temporalis*. Letters A–E mark localities listed in Table 1. The heavy lines partitioning Australia indicate the inland limit of the distribution of this species.

Four of the five species were exclusively of type L, whereas *P. temporalis* displayed only S (18/38 individuals or 47%) and M (20/38 or 53%) types. Individuals from southern populations of *P. temporalis* (localities A, B and C, Table 1) are exclusively of type S, whereas individuals from localities D and E are exclusively M (Figure 2).

Localization of length variation: Typical hybridizations of the srDNA PCR probe to Southern blots of babbler DNA are shown in Figure 3. Whereas the srRNA probe hybridized to fragments bearing length mutations (Figure 3A), the cytochrome *b* probe did not (not shown). As predicted, the srDNA probe hybridized to the 1.7-kb *Sac*II fragment spanning sites within the small and large mitochondrial rRNA genes (Figure 3B; CARR, BROTHERS and WILSON 1987). The pattern of hybridization of the 1-kb cytochrome *b* probe to blots of *temporalis*, *superciliosus* and *ruficeps* DNA cut with *Bam*HI, *Cla*I and *Hin*dIII was identical to that of the smaller cytochrome *b* probe (not shown).

mtDNA types 9 and 10 (*temporalis*, M and S, respectively), 17 (*superciliosus*), 20 (*ruficeps*), 22 (*isidori*) and 23 (*halli*) were chosen for restriction mapping with the eight enzymes listed in MATERIALS AND METHODS, which produced an average of 18 sites per map (Figure 4). In each map, the discretely varying fragment lay directly to the left of a *Cla*I (c or c*; Figure 4) site lying between the pair of conserved *Sac*II (s) sites known to lie within the small and large rRNA genes in diverse vertebrate mtDNAs (CARR, BROTHERS and WILSON 1987).

Direct sequencing of the probe produced by the srRNA primers (Figure 5) allowed alignment of the probe's map position with the above-described *Cla*I

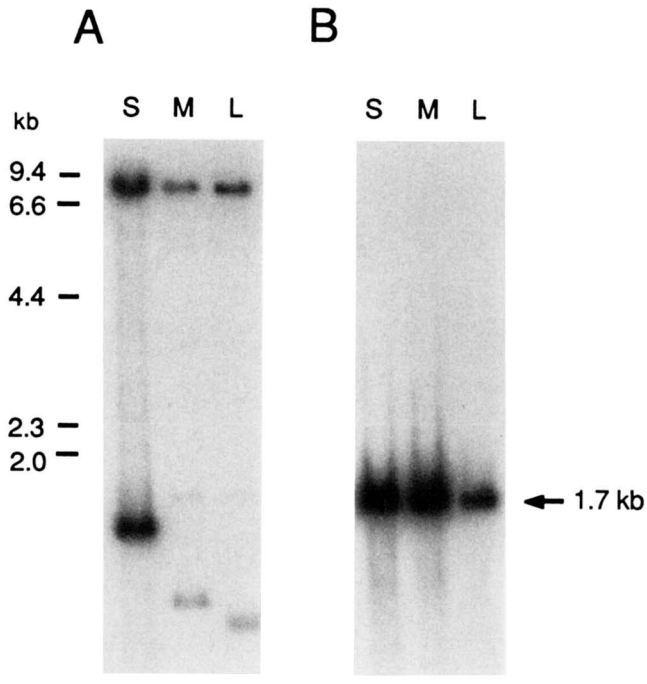

FIGURE 3.—Hybridization of a mitochondrial srDNA probe to Southern blots of babbler genomic DNA. S, M, and L indicate individuals scored as having small, medium, and large mtDNAs, respectively. A, detection of length mutations in fragments of DNA cut with *Cla*I/*Bam*HI. B, detection of the diagnostic 1.7-kb fragment (indicated with arrow) in DNA cut with *Sac*II.

site (Figure 4), which is found in this gene in all but one of the babbler mtDNAs (type 15; Table 6) and in a human sequence (ANDERSON *et al.* 1981). Furthermore, we found no *Eco*RI sites in our sequence analysis of a 0.9-kb cytochrome *b* segment (S. V. EDWARDS, P. ARCTANDER and A. C. WILSON, unpublished results), suggesting that this segment lies to the left of the *Eco*RI site closest to the region showing length variation in the *Pomatostomus ruficeps* map (e; Figure 4). Like many length variants described before in animal mtDNA, the one present in babbler mtDNA lies in a region between the cytochrome *b* and srRNA genes, presumably in or near the control region, a noncoding, rapidly evolving portion of the genome containing the heavy strand origin of replication (see SOLIGNAC, MONNEROT and MOUNOLOU 1983; HARRISON, RAND and WHEELER 1985; DENSMORE, WRIGHT and BROWN 1985; MORITZ and BROWN 1986, 1987). The pattern of hybridizations and alignments of the cytochrome *b* and srRNA probes with these restriction sites is compatible with the gene order and size of chicken mtDNA (DESJARDINS and MORAIS 1990), although experimental confirmation of this gene order in babbler mtDNA is still required.

mtDNA map variability

Restriction site variation within *P. temporalis*: Further mapping of 50 babbler mtDNAs identified a total of 53 different sites among 23 distinct types (Table 7 in APPENDIX). Only the two conserved *Sac*II sites defining the 1.7-kb fragment were found in all mtDNA types. Among the 15 mtDNA types detected within *P. temporalis* (Table 6), the restriction survey identified a total of 19 variable sites, ten of which were phylogenetically informative (Table 7). Parsimony analysis using both unordered and Dollo characters revealed two major clades of mtDNAs (Figure 6A), one consisting of individuals from northern

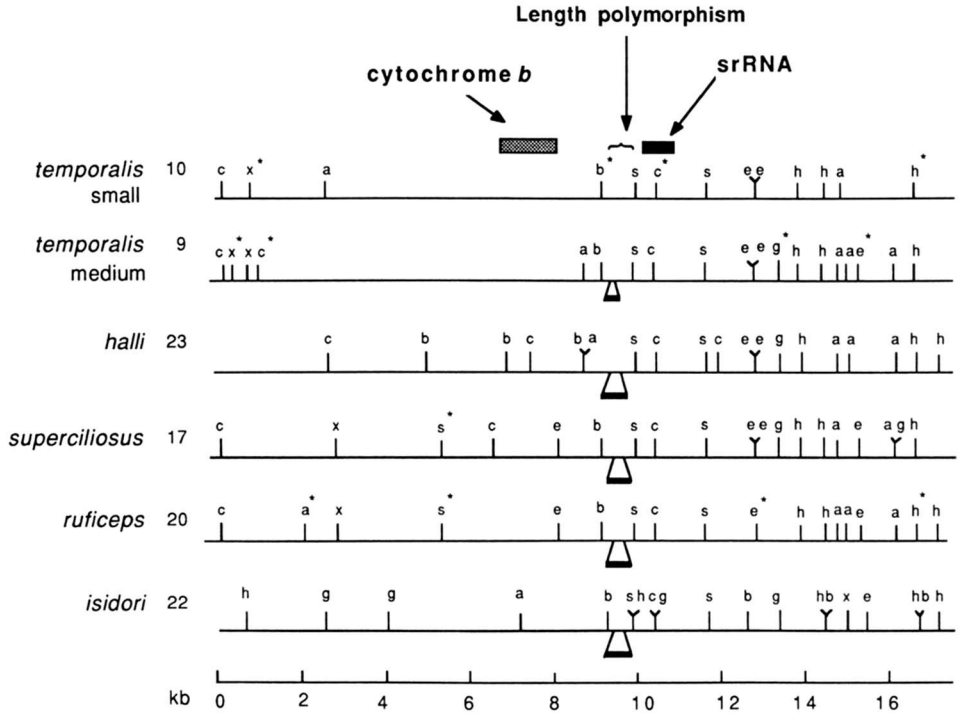

FIGURE 4.—Restriction maps of six types of babbler mtDNA. Scale in kilobases appears at the bottom. Numbers next to taxon names correspond to types listed in Table 6 in the APPENDIX; *P. temporalis* is represented by two maps, whose mtDNA size classes are indicated. Restriction enzymes are designated as follows: a, *Sal*I; b, *Bam*HI; c, *Cla*I; e, *Eco*RI; g, *Bgl*II; h, *Hin*dIII; s, *Sac*II; x, *Xba*I. Asterisks mark polymorphic sites within species, or within mtDNA size classes for *P. temporalis*. Insertions relative to the map for *temporalis* type 10 are indicated by solid bars below maps. Above all the maps, stippled and solid bars indicate positions of the large cytochrome *b* and srDNA probes, respectively (see MATERIALS AND METHODS). The region containing the length polymorphism is indicated. Two *Xba*I sites found only in the *superciliosus* mtDNAs (Table 7 in the APPENDIX) are not mapped; one *Bam*HI site unique to *halli* is not mapped.

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                                                                                                                ClaI
Babbler  TTGATGCTTTATCTTACCTGAGCATCCGCCCGAGAACTACGAGCACTAACGCTTAAAACTCTAAGGACTTGGCGGTGC-CCAAACCCACCTAGAGGAGCCTGTTCTATAATCGATAAT
Human    .CA.CAG..A.ATCA..AAA.CTGCT....A..AC.....-.-.CA.....A.....C.....TT..T.T..CT.....G.._____..A
                                                  1150                                  1200

Babbler  CCACGATACACCTGACCATCCCTCGCCCATAACAGCCTATATACCGCCGTCGCCAGTTCACCCCCCCTGAAGTCCAACAGTGAGCGCAATAGTCTCTCCCACTAATACGACAGGTCAA
Human    ..C....CA....C....--....TTG.-.--.....A..TT...-CA.A...TGA.....-G.T...AA.TAA..GCA...-A.C.A.GTA..G...TT.....
                                       1250                                  1300

Babbler  GGTATAGCCTATGGGATGG-AAGTAATGGGCTACATTTCCTAAATTAGAAAATCACGGCACAGGGGTTTGAAATTACCCCTAGAAGGCGGATTTAGCAGTAAAGTGGGATTATCGAG
Human    ...G.....C...A.G...C...A.....T...CCCC.....CT...AT.GCCCTTA.GA..C...AGGG.C.....T.....C.AA..G..GA.T.
                  1350                                  1400
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FIGURE 5.—Sequence of a 351-bp segment of the srRNA gene in the grey-crowned babbler, *Pomatostomus temporalis* (type 1) aligned with positions 1107–1449 of the human sequence (ANDERSON *et al.* 1981). The sequences were aligned with the Sellers' alignment program on the University of California, Berkeley, mainframe computer with a "gap penalty" of four. The alignment allowed the sequences to match at 237 positions; at the remaining 116 positions shown, there are 104 nucleotide differences and 10 deletions or additions totaling 12 bp. Numbers below the sequences indicate the positions in the human mtDNA. The *Cla*I site marked here in the babbler and human sequences appears in Figure 4 directly below the indicated position of the srDNA probe and is present in every map depicted there.

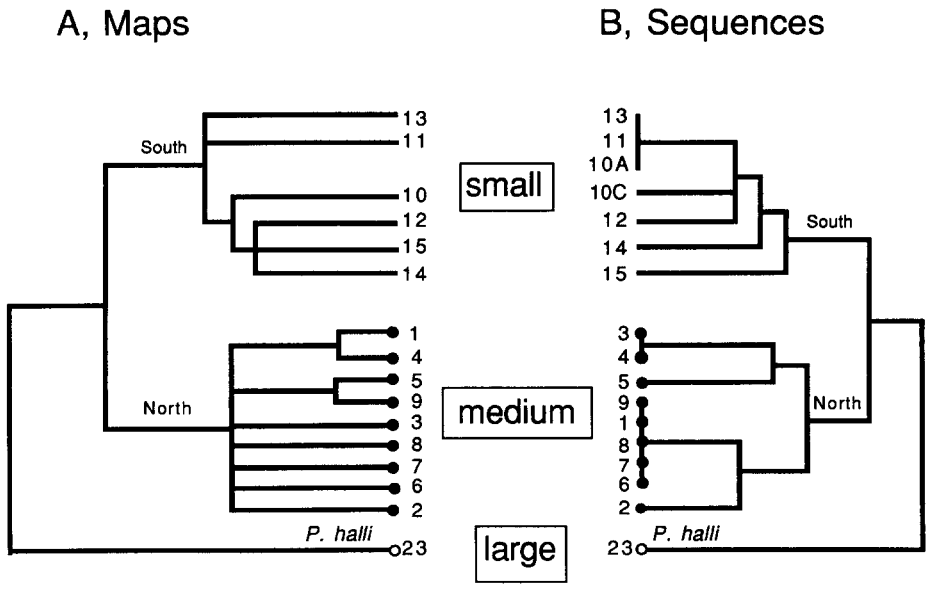

FIGURE 6.—Intraspecific trees relating mtDNAs of *P. temporalis* to those of *P. halli*. Numbers correspond to mitochondrial types listed in Table 6 in the APPENDIX; the letters A and C denote localities in Table 1. No circles at tips represent small mtDNAs, closed circles medium mtDNAs, and open circles large mtDNAs. South refers to mtDNAs from localities A–C (Table 1) and north to localities D and E. Both trees were rooted using *P. halli* type 23C as an outgroup. A, Majority-rule consensus of 500 equally parsimonious trees (19 steps) found in unordered parsimony analysis of the variable restriction sites listed in Table 7 in the APPENDIX. The tree search was aborted when 500 trees had been saved; most of these trees differed from one another in the placement of mtDNAs *within* the southern and northern clades as depicted in the figure. Branches leading to southern and northern types were found in 89% and 52% of 200 bootstrap replicates, respectively. B, Majority-rule consensus of five equally parsimonious trees (34 steps) relating 16 *P. temporalis* cytochrome *b* sequences. In 200 bootstrap replicates of the 13 informative sites (*P. halli* included), the indicated southern and northern clades were monophyletic 42% and 76% of the time, respectively. See Table 8 in the APPENDIX for further statistical tests.

Queensland and the Northern Territory (localities D and E, Table 1 and Figure 2), the other containing exclusively the individual from southern Queensland and individuals from New South Wales (localities A–C, Table 1 and Figure 2).

Using an improved method of estimating sequence divergence from restriction maps (WILSON *et al.* 1989), the maximum depth of the northern and southern clades corresponds to mtDNA sequence divergences of about 3.4% and 5.8%, respectively (Table 3; means within clades: 1.4% and 2.3%, respectively). Divergences between individuals of different clades are higher (mean, 8.2%). Because small mtDNA genomes were present exclusively in populations A–C, the northern and southern clades in the restriction tree of *temporalis* mtDNA types also correspond to the mtDNAs of medium and small lengths (Figure 6A). Thus the length polymorphism analyzed here constitutes a further case in which mtDNA length may serve as a reliable genetic tag among mtDNAs within and between species (*cf.* WRISCHNIK *et al.* 1987; STONEKING and WILSON 1989).

Restriction site variation among species: A total of 51 variable and 24 informative sites were detected among the 23 mtDNA types. Interspecific divergence estimates ranged from an average of 6.8% between *ruficeps* and *superciliosus* to an average of 52% in comparisons involving *P. isidori* (Table 3). In addition

TABLE 3

Pairwise comparison of 23 types of babbler mtDNA

MtDNA type	*temporalis*-north									*temporalis*-south						*halli*	*superciliosus*		*ruficeps*				*isidori*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	23	16	17	18	19	20	21	22
1	—	2.1	1.0	0.5	1.0	0.5	1.0	1.0	0.5	5.6	7.2	8.2	9.2	6.5	10.4	16.5	9.5	10.4	6.8	11.6	7.6	5.9	49.9
2	4	—	3.4	2.8	3.2	2.8	3.4	2.1	2.6	7.4	9.3	10.4	11.7	8.4	12.8	19.2	14.8	16.0	11.5	18.7	12.6	10.4	79.7
3	2	6	—	0.5	1.0	1.6	1.0	1.0	0.5	5.6	7.2	8.2	9.2	6.5	10.4	12.6	7.0	7.8	6.8	11.6	7.6	5.9	36.2
4	1	5	1	—	1.5	1.1	1.6	1.6	1.0	4.7	6.1	7.2	8.0	5.6	9.3	15.2	8.5	9.5	5.9	10.4	6.8	5.1	46.8
5	2	6	2	3	—	1.5	1.0	1.0	0.4	7.4	9.3	10.4	11.7	8.4	12.8	14.8	8.7	9.5	8.5	14.0	9.5	7.6	41.2
6	1	5	3	2	3	—	0.5	1.6	1.0	4.7	6.1	7.2	8.0	5.6	9.3	15.2	8.5	9.5	5.9	10.4	6.8	5.1	46.8
7	2	6	2	3	2	1	—	1.0	0.5	5.6	7.2	8.2	9.2	6.5	10.4	12.6	7.0	7.8	6.8	11.6	7.6	5.9	36.2
8	2	4	2	3	2	3	2	—	0.5	5.6	7.2	8.2	9.2	6.5	10.4	12.6	9.5	10.4	9.4	15.7	10.4	8.4	49.9
9	1	5	1	2	1	2	1	1	—	6.5	8.2	9.3	10.4	7.4	11.6	13.7	7.8	8.7	7.6	12.8	8.5	6.8	38.7
10	7	9	7	6	9	6	7	7	8	—	0.7	1.4	1.6	0.7	3.0	27.1	10.4	11.6	10.4	19.0	11.7	9.2	50.9
11	8	10	8	7	10	7	8	8	9	1	—	2.4	0.8	1.4	4.2	25.0	13.0	14.4	13.3	25.0	14.8	11.8	71.7
12	9	11	9	8	11	8	9	9	10	2	3	—	3.7	0.7	3.0	38.0	14.4	15.8	14.8	19.0	16.4	13.3	77.0
13	9	11	9	8	11	8	9	9	10	2	1	4	—	2.4	5.8	22.9	11.7	13.0	11.8	22.7	13.3	10.4	66.7
14	8	10	8	7	10	7	8	8	9	1	2	1	3	—	2.0	29.3	11.6	12.8	11.7	21.0	13.0	10.4	54.7
15	11	13	11	10	13	10	11	11	12	4	5	4	6	3	—	43.8	17.2	18.7	18.0	32.5	19.7	16.4	88.0
23	16	18	14	15	16	15	14	14	15	17	16	19	15	18	21	—	25.0	26.7	20.2	33.9	21.8	18.7	79.7
16	12	16	10	11	12	11	10	12	11	11	12	13	11	12	15	20	—	0.4	6.2	10.4	7.0	5.4	41.2
17	13	17	11	12	13	12	11	13	12	12	13	14	12	13	16	21	1	—	5.0	8.5	5.8	6.2	43.8
18	9	13	9	8	11	8	9	11	10	10	11	12	10	11	14	17	9	8	—	1.8	0.5	0.5	33.9
19	12	16	12	11	14	11	12	14	13	13	14	13	13	14	17	20	12	11	3	—	1.1	2.6	43.8
20	10	14	10	9	12	9	10	12	11	11	12	13	11	12	15	18	10	9	1	2	—	1.1	36.2
21	8	12	8	7	10	7	8	10	9	9	10	11	9	10	13	16	8	9	1	4	2	—	31.5
22	23	27	21	22	23	22	21	23	22	20	21	22	20	21	24	27	23	24	20	21	21	19	—

For each pair of mtDNAs, the percent sequence divergence, estimated by the method described in WILSON *et al.* (1989), appears above the diagonal and the number of restriction site differences below the diagonal.

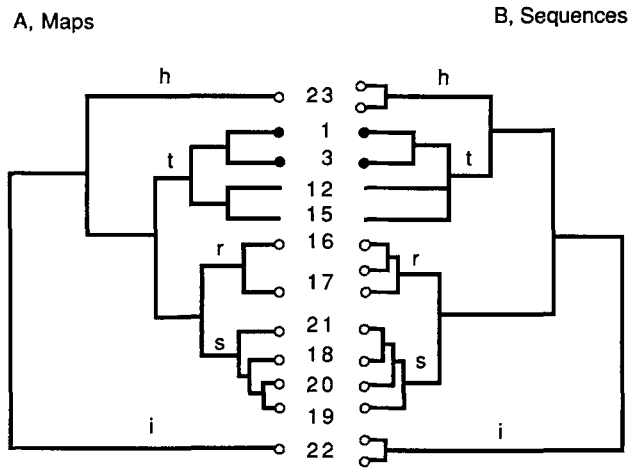

FIGURE 7.—Interspecific trees relating mitochondrial types from the five species of babblers (h = *halli*; i = *isidori*; r = *ruficeps*; s = *superciliosus*; t = *temporalis*). Both trees were rooted with the mtDNA of the New Guinean *P. isidori* (i). Open circles, closed circles, and bare branch tips indicate large, medium, and small mtDNA size classes, respectively. A, The single most parsimonious tree (67 steps) based on unordered parsimony analysis of the restriction site data in Table 7 in the APPENDIX. In 3 of 7 equally parsimonious trees produced by Dollo parsimony *halli* is closest to northern *temporalis* types 1 and 3, causing *temporalis* to be paraphyletic (not shown; see Table 8, APPENDIX). B, Consensus of two equally parsimonious trees (94 steps) relating 15 cytochrome *b* sequences. The numbers refer to the mtDNA types in Table 6 in the APPENDIX. All trees except those in which *halli* falls within *temporalis* support the hypothesis of a single origin of the large mitochondrial genome size followed by two successive deletions leading to the medium and small genomes present in *temporalis* (see DISCUSSION).

to the eight mtDNA types detected in species other than *temporalis*, four divergent *temporalis* types indicated by the cytochrome *b* sequence analysis (types 1, 3, 12 and 15; see below) were chosen to represent this species in interspecific analyses. Both the unordered and Dollo analyses suggest that *ruficeps* and *superciliosus* are mitochondrially sister species (Figure 7A), though this association is not significant based on the map data alone (see Table 8 in APPENDIX). The placement of *halli* shifts among the trees, in some cases causing the *temporalis* lineage to be paraphyletic (see legend, Figure 7).

Cytochrome *b* sequences

Variability among babblers: Figure 8 shows the sequences of the 282-bp portion of the cytochrome *b* gene determined for 27 mtDNAs representing each of the 23 types defined by restriction analysis (Tables 3 and 6). No deletions or additions were found in this segment of cytochrome *b* relative to the human sequence (ANDERSON *et al.* 1981). A total of 64 positions were variable among the 20 different sequences, 62 of which were phylogenetically informative. In three cases involving ten mtDNAs, the short cytochrome *b* sequences failed to discriminate where restriction analysis had (*e.g.*, types 1E and 6–9D, Table 4). However, in each of the four cases in which multiple individuals were sequenced for a single mtDNA type defined by restriction analysis, further discrimination of the type was evident (*e.g.*, types 10A and 10C, Table 4).

Sequence variation within *P. temporalis*: A total of 17 positions were variable among the 16 sequences (9 distinct sequences) from *P. temporalis*. Uncorrected sequence differences (Table 4) ranged from none (0%; *e.g.*, types 7 and 8) to 11 differences (3.9%; types 2 and 5 *vs.* type 15; Table 4). Mean uncorrected pairwise differences between mtDNA types within northern and southern groups averaged 1.4% and 1.3%, respectively, with an average of 3.2% sequence difference between types from individuals of different geographic clades (Table 4). All of the substitutions within *temporalis* are silent (Table 4). Phylogenetic analysis of the 12 informative sites among the nine distinct *temporalis* sequences yielded five equally parsimonious trees (Figure 6B), the consensus of which agreed with those produced by restriction enzymes (Figure 6A) in that there are two major clades corresponding to the geographically segregated small and medium genome sizes. Trees indicating monophyly of both northern and southern sequences were always superior, frequently at a statistically significant level, to those in which one or both of these clades were not monophyletic (Table 8).

Sequence variation among species: Uncorrected percent differences for this segment of cytochrome *b* within species other than *temporalis* ranged from 0.4 to 2.1% (mean number of differences = 2.8, or 1.0%), and differences among species ranged from a mean of 6.2% between *halli* and *temporalis* to 12.0% between *isidori* and other species (Table 4). The consensus tree produced by analysis of 61 informative positions among 15 sequences (Figure 7B) agrees with the restriction tree in Figure 7A in that *Pomatostomus superciliosus* and *ruficeps* appear as sister species; the sequences suggest strong support for their association relative to *temporalis* and *halli* by winning-sites analyses (Table 8). *P. halli*, which appears as the sister species to *temporalis* in all the trees derived by parsimony analysis of the cytochrome *b* sequences (Figure 7B), would have been expected on the basis of plumage patterns to be derived from *superciliosus* (FORD 1974; HALL 1974).

Combined map and sequence analysis: When combined, the restriction maps and cytochrome *b* sequences discerned a total of 27 mtDNA types among the five species, with 115 variable sites. Combining the data without modification is justified here, since in no case was a restriction site found to lie within the 282-bp piece of cytochrome *b* that was sequenced, and thus no sites in the combined data set were redundant. Parsimony analysis using all 27 types with both *P.*

TABLE 4

Comparisons of 27 cytochrome *b* sequences

MtDNAs compared	*temporalis*									*halli*		*superciliosus*			*ruficeps*				*isidori*	
	1E	2E	3E	5E	10C	10A	12B	14B	15A	23C	23J	16A	17G	17F	18F	19B	20F	21B	22I	22I
1E, 6D–9D	—	0	0	0	0	0	0	0	0	1	1	2	2	2	0	1	0	0	8	8
2E	3	—	0	0	0	0	0	0	0	1	1	2	2	2	0	1	0	0	8	8
3E, 4E	6	5	—	0	0	0	0	0	0	1	1	2	2	2	0	1	0	0	8	8
5E	5	4	1	—	0	0	0	0	0	1	1	2	2	2	0	1	0	0	8	8
10C	10	9	10	9	—	0	0	0	0	1	1	2	2	2	0	1	0	0	8	8
10A, 11B, 13B	9	8	9	8	1	—	0	0	0	1	1	2	2	2	0	1	0	0	8	8
12B	10	9	8	7	2	1	—	0	0	1	1	2	2	2	0	1	0	0	8	8
14B	8	7	8	7	2	1	2	—	0	1	1	2	2	2	0	1	0	0	8	8
15A	10	11	10	11	8	7	8	6	—	1	1	2	2	2	0	1	0	0	8	8
23C	16	19	20	19	14	15	16	16	18	—	0	1	1	1	1	2	1	1	7	7
23J	17	20	21	20	15	16	17	17	19	1	—	1	1	1	1	2	1	1	7	7
16A	23	24	25	24	21	22	23	21	23	23	24	—	0	0	2	1	2	2	8	8
17G	24	25	26	25	22	23	24	22	24	24	25	1	—	0	2	1	2	2	8	8
17F	23	24	25	24	21	22	23	21	23	25	24	4	3	—	2	1	2	2	8	8
18F	25	26	25	24	27	26	25	25	25	29	28	22	23	20	—	1	0	0	8	8
19B	23	24	23	24	25	24	25	23	21	27	26	20	21	18	6	—	1	1	9	9
20F	24	25	24	23	26	25	24	24	24	28	27	23	24	21	1	5	—	0	8	8
21B	26	27	24	25	28	27	26	26	24	30	29	23	24	21	1	5	2	—	8	8
22I	34	35	32	33	34	35	34	36	34	34	33	35	36	33	33	31	32	32	—	0
22I	34	35	32	33	34	35	34	36	34	34	33	35	34	31	35	33	34	34	2	—

For each pair of sequences, numbers below the diagonal are the total number of observed differences (transitions and transversions), and numbers above the diagonal the observed number of replacement substitutions. Identical sequences appear on the same line, making 20 distinct sequences for this 282-bp segment (Fig. 8). Letters next to mtDNA type numbers indicate localities from which types were chosen for sequencing (see Tables 1 and 6).

isidori individuals as outgroups (Table 4) yielded 16 equally parsimonious trees. The consensus of these trees agreed with those based on cytochrome *b* sequences (Figures 6B and 7B), and the differences among the 16 trees were within each of the two clades within *temporalis*. Bootstrap analysis indicated that the northern and southern clades of *temporalis* were supported 81% and 90% of the time, respectively. Combining the data sets also provided increased power of discrimination among alternative interspecific hypotheses (Table 8), but still did not provide enough data to discriminate at a statistically significant level between monophyly and paraphyly of *temporalis* mtDNAs relative to *halli* mtDNAs (interspecific tests, Table 8).

DISCUSSION

Phylogeny and coalescence

There is growing interest in using coalescent theory to analyze population processes (see TAVARÉ 1984; TAKAHATA and NEI 1985). In the study of intraspecific polymorphisms (in this case the length polymorphism in *P. temporalis* mtDNA), coalescent theory makes it profitable to know the genealogy and extent of divergence of alleles found both within the population (or species) under study as well as in closely related populations or species (see TAKAHATA 1989). Furthermore, the introduction of statistical testing into phylogenetic analysis makes it possible not only to estimate the number and direction of length mutations that have arisen in surviving *temporalis* lineages but also to provide a framework in which coalescent theory can be applied. In the following sections we use the coalescent approach to estimate rates of gene flow and mutation that are consistent with the geography and phylogenetic distribution of the length variation studied here.

Utility of phylogenetic analysis: Analysis of the phylogenetic distribution of three length variants in babbler mtDNA has allowed us to propose that the two smaller size classes present in *P. temporalis* populations arose from a third, larger class present exclusively in the other four species. Only by screening for length variation outside *P. temporalis* were we able to establish the evolutionary direction of the length variation in this taxon; as few as two successive deletion events in the past can account for the observed length polymorphism present. Directional trends for mtDNA genome size are not without precedent (SOLIGNAC, MONNEROT and MOUNOLOU 1986; BOYCE, ZWICK and AQUADRO 1989) and have been visualized only in light of phylogenetic analysis.

Restricted gene flow: SLATKIN (1989) found that patterns of mtDNA variation in which there was complete concordance of the mtDNA phylogeny with geography were consistent with an hypothesis of low levels of gene flow between the geographic regions under consideration. The phylogeny of mtDNA

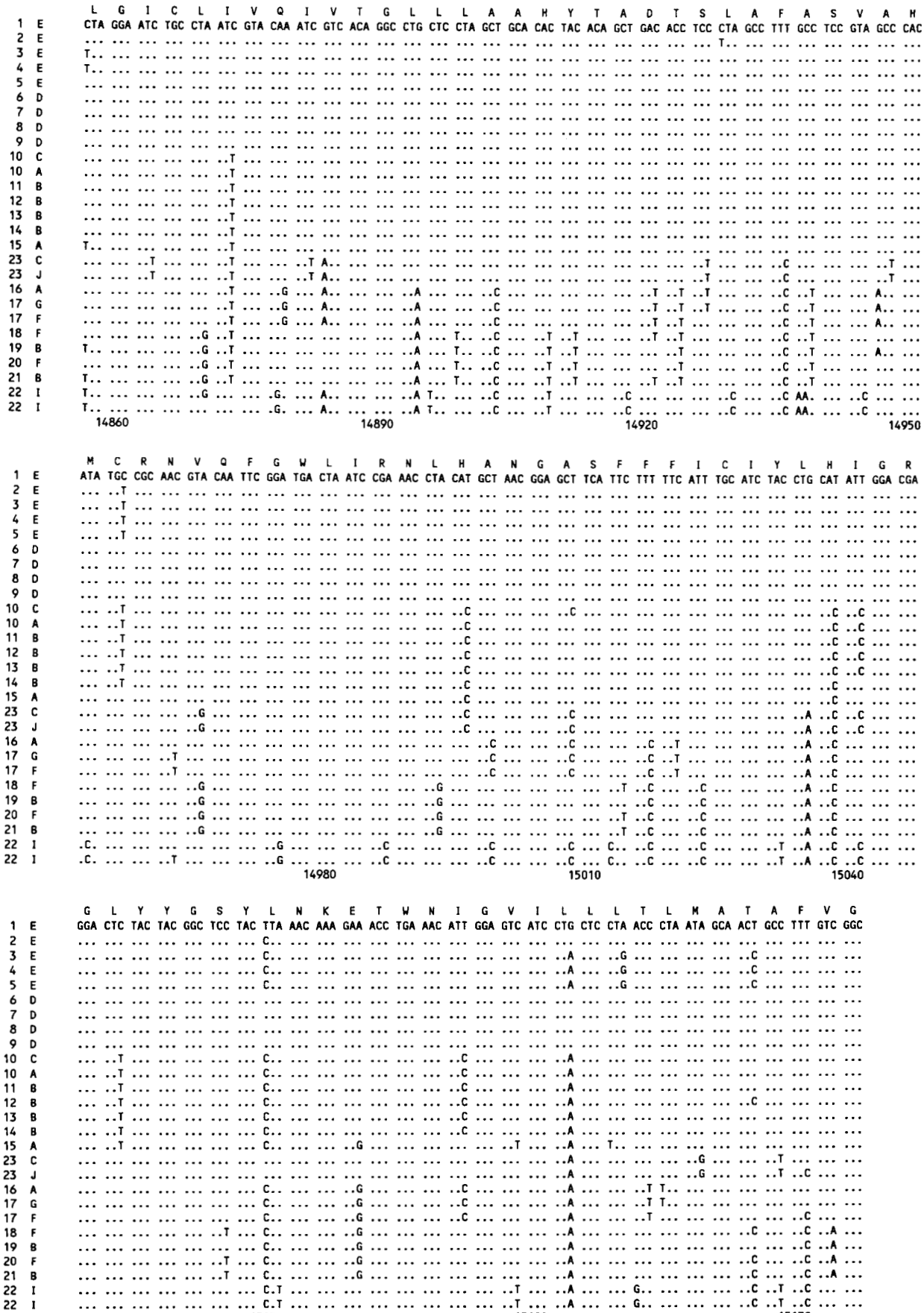

FIGURE 8.—Sequences of a 282-bp segment of cytochrome *b* from 27 babbler mtDNAs. The nucleotide sequence and inferred amino acid

length and site differences within *P. temporalis* conforms to such a pattern, and the branch separating northern and southern clades is statistically supported. Although the sample sizes required to reject an hypothesis of high gene flow (>1 migrant/generation) are met by the restriction data but not by the sequences (SLATKIN 1989), we view these data as consistent with an hypothesis of very low gene flow between the northern and southern populations sampled in this study. The estimate of G_{st} (NEI 1987), or the fraction of the total diversity apportioned among *temporalis* populations, is 0.17, a value that would suggest a moderate level of gene flow among the five populations sampled. However, we agree with SLATKIN and MADDISON (1989) that in taxa such as *P. temporalis*, in which the number of alleles relative to the sample size is large and their frequencies per locality small, analysis of geographic variation using G_{st} is inadequate for estimating historic levels of gene flow.

The geographic pattern of mtDNA length and site variation within *P. temporalis* reflects a major biogeographic break running southeast from the Gulf of Carpenteria and thought to underlie speciation patterns in other taxa across this region (barrier B in Figure 9 of CRACRAFT 1986; see KEAST 1961, 1981; SCHODDE and CALABY 1972). The degree of mtDNA divergence (Tables 3 and 4) and the number of diagnostic or nearly diagnostic sites for the taxa on either side of this break (Table 7, Figure 8) is large for subspecies of birds (AVISE and ZINK 1988), even when our values for restriction data (Table 3) are corrected to match the methods used to calculate per cent divergence in earlier studies (not shown). These large mtDNA differences between northern and southern localities warrant further investigation into the specific status of *P. t. temporalis* and *P. t. rubeculus* (*cf.* Royal Australasian Ornithologists' Union 1926).

The distribution of shared mtDNA types among families within the northern and southern clades is consistent with there being little genetic structuring imposed by family groups within *P. temporalis*. Of the six family groups of this species whose mtDNAs were characterized for multiple individuals in the restriction survey, four possessed multiple mtDNA types (Table 5). Furthermore, at the level of resolution offered by the restriction maps and sequences, some widely separated families possessed a mtDNA type in common (Table 6), *e.g.*, type 14 in several families in localities A and B. While the conclusion based on this pattern is consistent with electrophoretic studies in this and other cooperatively breeding species (JOHNSON and BROWN 1980; MUMME *et al.* 1985), it needs to be verified with larger sample sizes from multiple localities. In summary, the geographic distribution of the S and M length variants is likely determined primarily by the pattern of high gene flow within northern and southern regions occupied by *P. temporalis* and low gene flow between them.

Rate of length mutation estimated by coalescent analysis: Coalescent theory provides a means of estimating the apparent mutation rate of the length variants from their underlying phylogenetic distribution. Additionally, our statistical evaluation of the phylogenies provides the value for a parameter required for application of coalescent theory. Figure 9 shows the setting in which coalescent theory can be applied to the pattern of length variation within *P. temporalis*. Under the assumption of neutrality, the mutation rate alone, μ, is difficult to estimate without some knowledge of the effective population size of mtDNA (N_f), but their product, $N_f\mu$, is more tractable. We can approach estimates of the upper bound of $N_f\mu$ by noting that in Figure 9, the mtDNA length does not change during time t_1 with a probability (P_0). P_0 is analogous to the probability that the n individuals with small mtDNAs are a monophyletic group–namely the statistical confidence of the branch leading to the small mtDNAs, or at best about 0.99 (Table 8). Assuming that mutation of mtDNA length occurs according to a Poisson distribution, P_0 can also be defined as $e^{-\mu T_n}$, where T_n is the total time in generations in the phylogeny under consideration (N. TAKAHATA, personal communication). T_n is the sum of the individual coalescence times (t_i) of the n alleles sampled; coalescent theory predicts that the distribution of t_i is an exponential function and is determined by N_f. This relationship causes t_i to drop out of equations relating P_0 and $N_f\mu$. Such an equation derived for a haploid population from these assumptions is (EWENS 1979):

$$P_0 = \prod_{k=1}^{n-1} (1/[1 + 2N_f\mu/k]), \quad (1)$$

where n is the number of alleles sampled in the clade diverging at time t_1 (Figure 9). For small values of $N_f\mu$, P_0 can be approximated as

$$1 - [1 + \tfrac{1}{2} + \tfrac{1}{3} + \cdots + 1/(n-1)]2N_f\mu. \quad (2)$$

Substituting for $n = 18$ and $P_0 = 0.99$ yields an estimate of the upper bound of $N_f\mu < 1.5 \times 10^{-3}$.

This value can be contrasted with the corresponding range of values estimated using frequency-based population genetic theory (BIRKY, FUERST and MA-

sequence of *P. temporalis* type 1 is at the top. For other sequences, only differences from type 1 are shown, with identity indicated by a dot. Number/letter combinations refer to types/localities from which individuals were chosen for sequencing (Table 6 in the APPENDIX). Numbers at the bottom give positions in accordance with the human sequence (ANDERSON *et al.* 1981). The sequences (positions 14885–15123) of individuals 1E, 17F, 18F and the first of the two 22I types are here repeated from KOCHER *et al.* (1989); the sequence reported for 22I at positions 15109–15110 in KOCHER *et al.* (1989) is here corrected.

TABLE 5

Estimates of diversity (h) within family groups of *P. temporalis*

Family number	Locality[a]	Number of mtDNA types	Number sampled (n)	h
10	B	2	3	0.67
11	B	3	3	1.0
12	B	1	2	0.0
13	E	3	3	1.0
22	D	3	3	1.0
23	D	1	4	0.0

Calculated according to the equation $h = (1 - \sum x_i^2)n/(n - 1)$, where x_i is the frequency of the ith mtDNA type.

[a] See Table 1.

RUYAMA 1989) for *Gryllus* crickets. To estimate μ, RAND and HARRISON (1989) used diversity indexes of three mtDNA size classes within and between individuals of *Gryllus firmus* and a field estimate of about 10^3 for N_f, and found that $\mu = 10^{-4}$; these values yield a rough estimate of $N_f\mu$ of 10^{-1}. The value of $N_f\mu$ for *P. temporalis* estimated in a phylogenetic context is thus approximately 100 times lower than that estimated for crickets. While we have no reliable estimate of N_f for *P. temporalis* at this time, many birds have estimated effective population sizes on the order of 10^2-10^3 (BARROWCLOUGH 1980, 1983; BARROWCLOUGH and SHIELDS 1984). This value would yield a range of estimates of μ that are 10–100 times lower than that estimated for crickets using frequency-based population genetic theory.

Our estimate of a lower bound on $N_f\mu$ (based on the probability that one medium-to-small mutation takes place during time t_2; Figure 9) is hampered by our lower confidence in the monophyly of *temporalis* small and medium mtDNAs as a whole (Table 8). However, the upper bound estimate of $N_f\mu$ is also consistent with the observation of two deletion events along branches about 12 times longer than t_1 (N. TAKAHATA, personal communication), a relative time that is consistent with the interspecific mtDNA divergences of lineages on which these deletions occur (Table 4, Figure 9).

This method of calculating $N_f\mu$ assumes that the rate of length mutation occurs according to a Poisson distribution and that all alleles are neutral; neither idea is necessarily correct (GILLESPIE 1986), particularly when applied to intraspecific distributions of mtDNA length variants, for which selection on small genome size has been invoked (MORITZ, DOWLING and BROWN 1987; RAND and HARRISON 1989). In addition, the order of magnitude of the resulting value of $N_f\mu$ is sensitive to the probabilities of there being no change during t_1. However, our preliminary result is quite consistent with the contrast in patterns of inter- and intraspecific distribution of three mtDNA size classes analyzed here and in *G. firmus*, the latter

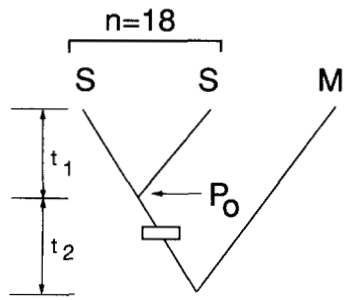

FIGURE 9.—Schematic framework for estimating mutation rate of length differences in *P. temporalis* mtDNA. The hollow bar denotes the deletion event converting medium to small mtDNAs. P_0 represents the probability that no length-mutational events took place during t_1. The $n = 18$ represents the number of *P. temporalis* mtDNAs scored as belonging to the small length class. See text for discussion.

of which showed extensive heteroplasmy and within-population variation for mtDNA size (RAND and HARRISON 1989). Although the location of the length variation in the control region needs to be confirmed by sequencing studies, our estimate of $N_f\mu$ would suggest a mutation rate much lower than that expected for base substitution in the control region (VIGILANT *et al.* 1989). A mutation rate lower than those responsible for most length polymorphisms in the control region (BERMINGHAM, LAMB and AVISE 1986; HALE and SINGH 1986; BOURSOT, YONEKAWA and BONHOMME 1987; RAND and HARRISON 1989; BUROKER *et al.* 1990) is consistent with both the lack of heteroplasmy and the high degree of geographic structure within *P. temporalis*.

Dynamics of cytochrome *b* evolution

The evolutionary dynamics of the bird cytochrome *b* sequences presented here are similar to those described for functional mitochondrial protein-coding genes in other vertebrates (WILSON *et al.* 1985). The differences between the most similar sequences show an extreme bias toward transitions (Figure 10). This pattern is underscored by the observation that, within all species as well as among *temporalis*, *superciliosus*, and *halli*, exclusively transitions occur, with transversions accumulating only in interspecific comparisons involving *ruficeps* and *isidori* (Figures 8 and 10). The bias for transitions displayed by this segment of babbler cytochrome *b* extends observations of the behavior of this gene in a smaller number of individuals (KOCHER *et al.* 1989). Although pairwise analysis of the cytochrome *b* sequences suggests that the process of cytochrome *b* evolution involves a transition/transversion ratio of approximately 20 to 1 (Figure 10), in tracing the most parsimonious patterns of nucleotide substitution along phylogenetic trees (*e.g.*, Figure 6, A and B), one observes well over 20 transitions before the first transversion. In agreement with this possibility, we note that the number of observed transitions in this segment of cytochrome *b* is already in the

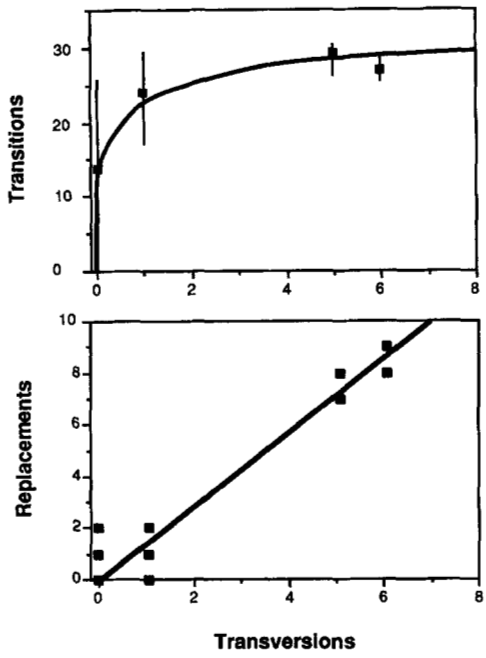

FIGURE 10.—Patterns of nucleotide substitution among 27 babbler cytochrome *b* sequences in Figure 8. The upper panel plots the average number of transitions per transversion drawn from a matrix of pairwise comparisons of the 20 distinct sequences among the 27 mtDNAs sequenced. Vertical lines represent absolute *ranges* of the number of transitions in each transversion class; solid boxes represent means within classes of comparisons with different numbers of transversions. The lower panel compares replacement substitutions *vs.* transversions for the 20 distinct sequences, with pairwise values for replacements drawn from Table 4.

multiple-hit zone within this songbird genus (Figure 10).

Most of the variation in this segment occurs in the third position of codons (49 of 64 sites or 77%; Figure 8). The observed proportion of variable sites by position among the 94-codon sequences corresponds to approximately 13%, 3% and 52%, respectively, of first, second and third positions. The relative variability at these positions is typical for other mitochondrial protein-coding genes studied (*e.g.*, BROWN *et al.* 1982; THOMAS and BECKENBACH 1989). Thus, despite the fact that transitions as a group may enter the multiple-hit zone within this genus, the third position transitions are likely the first to enter this zone. By contrast, the rate of replacement substitution in the cytochrome *b* segment is very low. This bias for silent substitutions is consistent both with the transition bias (since most synonymous substitutions are transitions) and with the low ratio of second to third position changes; indeed, replacements accumulate as slowly as do transversions (Figure 10). Although informative, the number of silent substitutions displayed by this short segment of cytochrome *b* alone is too small to be of rigorous use in studying within-region or within-population variation, and longer or more variable mtDNA sequences (*e.g.*, in the control region) are likely to prove more valuable for this purpose.

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APPENDIX

The APPENDIX consists of Tables 6–8, which provide details of fragment patterns and their distribution for 23 types of babbler mtDNA defined by restriction enzyme analysis (Table 6), the presence or absence of 51 variable restriction sites among these 23 types of mtDNA (Table 7), and statistical tests comparing alternative phylogenetic networks for four or more mtDNAs (Table 8).

TABLE 6

Fragment patterns and distribution of mtDNA types within and among babbler species

MtDNA type	Composite genotype[a]	Collecting localities and number of individuals[b]	Family number(s)[c]
temporalis			
1	AACADCBO	E:1	13
2	ACDCDABO	E:1	17
3	AACADCAA	E:1	13
4	AACADCAO	E:1	13
5	AACADCBB	D:2; E:4	D:19, 21; E:14–16, 18
6	AAAADCBO	D:1	22
7	AAAADCBA	D:1	22
8	AACADABA	D:1	22
9	AACADCBA	D:5	20, 23
10	AAAABAAO	A:2; B:3; C:1	A:3, 7; B:9, 12; C:24
11	AOAABAAO	B:2	10, 11
12	FAAAAAAO	B:1	11
13	AOOABAAO	B:1	11
14	AAAAAAAO	A:4; B:2	A:1, 2, 6, 8; B:10
15	AAAAAADO	A:1	4
superciliosus			
16	AABAFECC	A:1	30
17	AABBFECC	F:1; G:1	34, 38
ruficeps			
18	BAFBCBAO	C:1; F:2; H:2	C:57; F:52, 54; H:55, 56
19	CAFBEDAO	B:1	45
20	BAFBEBAO	F:1	49
21	BAFACBAO	B:1	48
isidori			
22	DDEAGFED	I:2	58, 59
halli			
23	EEOADAFA	C:1; J:1	39, 42

[a] Capital letters refer to fragment patterns for each enzyme in the following order: *Hin*dIII, *Bam*HI, *Xba*I, *Sac*II, *Sal*I, *Eco*RI, *Cla*I, *Bgl*II. "O" means no sites for that mtDNA type digested with that enzyme.

[b] Letters refer to localities (see Table 1), followed by the number of individuals.

[c] See MATERIALS AND METHODS. Where appropriate, capital letters indicate specific localities (Table 1) of families listed. Family group analysis is part of a larger study and numbering is not necessarily consecutive within or between species.

TABLE 7

Fifty-one variable sites among 23 babbler mtDNA types defined by restriction analysis

MtDNA type	Presence of site							
	*Hin*dIII	*Bam*HI	*Xba*I	*Sac*II	*Sal*I	*Eco*RI	*Cla*I	*Bgl*II
1	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	1 1 0 0 0 0 0	0 0	0 0 0 0 1 1 1 1	0 1 1 1	1 1 0 0 0 0 0 1 0	0 0 0 0 0 0
2	0 0 1 1 1 0	0 0 0 1 1 0 0 0 0	1 1 1 0 0 0 0	1 0	0 0 0 0 1 1 1 1	0 1 1 0	1 1 0 0 0 0 0 1 0	0 0 0 0 0 0
3	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	1 1 0 0 0 0 0	0 0	0 0 0 0 1 1 1 1	0 1 1 1	1 0 0 0 0 0 0 1 0	0 0 0 1 0 0
4	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	1 1 0 0 0 0 0	0 0	0 0 0 0 1 1 1 1	0 1 1 1	1 0 0 0 0 0 0 1 0	0 0 0 0 0 0
5	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	1 1 0 0 0 0 0	0 0	0 0 0 0 1 1 1 1	0 1 1 1	1 1 0 0 0 0 0 1 0	0 0 0 1 0 1
6	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	0 1 0 0 0 0 0	0 0	0 0 0 0 1 1 1 1	0 1 1 1	1 1 0 0 0 0 0 1 0	0 0 0 0 0 0
7	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	0 1 0 0 0 0 0	0 0	0 0 0 0 1 1 1 1	0 1 1 1	1 1 0 0 0 0 0 1 0	0 0 0 1 0 0
8	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	1 1 0 0 0 0 0	0 0	0 0 0 0 1 1 1 1	0 1 1 0	1 1 0 0 0 0 0 1 0	0 0 0 1 0 0
9	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	1 1 0 0 0 0 0	0 0	0 0 0 0 1 1 1 1	0 1 1 1	1 1 0 0 0 0 0 1 0	0 0 0 1 0 0
10	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	0 1 0 0 0 0 0	0 0	0 1 0 0 0 1 0 0	0 1 1 0	1 0 0 0 0 0 0 1 0	0 0 0 0 0 0
11	0 0 1 1 1 0	0 0 0 0 0 0 0 0 0	0 1 0 0 0 0 0	0 0	0 1 0 0 0 1 0 0	0 1 1 0	1 0 0 0 0 0 0 1 0	0 0 0 0 0 0
12	0 0 1 1 0 0	0 0 0 1 0 0 0 0 0	0 1 0 0 0 0 0	0 0	0 1 1 0 0 1 0 0	0 1 1 0	1 0 0 0 0 0 0 1 0	0 0 0 0 0 0
13	0 0 1 1 1 0	0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0	0 0	0 1 0 0 0 1 0 0	0 1 1 0	1 0 0 0 0 0 0 1 0	0 0 0 0 0 0
14	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	0 1 0 0 0 0 0	0 0	0 1 1 0 0 1 0 0	0 1 1 0	1 0 0 0 0 0 0 1 0	0 0 0 0 0 0
15	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	0 1 0 0 0 0 0	0 0	0 1 1 0 0 1 0 0	0 1 1 0	1 0 0 0 0 1 1 0 0	0 0 0 0 0 0
16	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	0 0 0 1 1 1 0	0 0	0 0 0 0 0 1 0 1	1 1 1 1	1 0 0 1 0 0 0 1 0	0 0 0 1 1 0
17	0 0 1 1 1 0	0 0 0 1 0 0 0 0 0	0 0 0 1 1 1 0	0 1	0 0 0 0 0 1 0 1	1 1 1 1	1 0 0 1 0 0 0 1 0	0 0 0 1 1 0
18	0 0 1 1 1 1	0 0 0 1 0 0 0 0 0	0 0 0 1 0 0 0	0 1	0 0 0 0 0 1 1 1	1 1 0 1	1 0 0 0 0 0 0 1 0	0 0 0 0 0 0
19	0 0 1 1 0 1	0 0 0 1 0 0 0 0 0	0 0 0 1 0 0 0	0 1	1 0 0 0 0 1 1 1	1 0 0 1	1 0 0 0 0 0 0 1 0	0 0 0 0 0 0
20	0 0 1 1 1 1	0 0 0 1 0 0 0 0 0	0 0 0 1 0 0 0	0 1	1 0 0 0 0 1 1 1	1 1 0 1	1 0 0 0 0 0 0 1 0	0 0 0 0 0 0
21	0 0 1 1 1 1	0 0 0 1 0 0 0 0 0	0 0 0 1 0 0 0	0 0	0 0 0 0 0 1 1 1	1 1 0 1	1 0 0 0 0 0 0 1 0	0 0 0 0 0 0
22	1 1 0 1 1 1	0 0 0 1 0 0 1 1 1	0 0 0 0 0 0 1	0 0	0 0 0 1 0 0 0 0	0 0 0 1	0 0 0 0 0 0 0 1 0	1 1 1 1 0 0
23	0 0 1 0 1 1	1 1 1 0 0 1 0 0 0	0 0 0 0 0 0 0	0 0	0 0 0 0 1 1 1 1	0 1 1 0	0 0 1 0 1 0 0 1 1	0 0 0 1 0 0
	* *	†	† * * * * *	*	* † † † † †	* * * †	* † *	† *

For each enzyme the variable sites are arranged proceeding approximately in the 5′ to 3′ direction along the mtDNA light strand (see also Fig. 4). The presence and absence of each site in each type of mtDNA are indicated by 1 and 0, respectively. In the last row of the table, daggers (†) mark the ten sites that are phylogenetically informative among *P. temporalis* mtDNAs (types 1–15), while asterisks (*) mark the 14 sites which are informative only when mtDNAs from the other species are also considered. Sizes of fragments for each type and enzyme are available from S.V.E.

TABLE 8

Examples of statistical tests comparing alternative phylogenetic trees

Network or tree description			Number of sites favoring alternative trees								
			Restriction maps			Sequences			Combined data		
A	B	C	A	B	C	A	B	C	A	B	C
Within *P. temporalis*: north vs. south											
1. 1/3 *vs.* 12/15	1/12 *vs.* 3/15	1/15 *vs.* 3/12	7	0†	0†	4	1	2	11	1†	2*
2. 2/6 *vs.* 10C/11	6/11 *vs.* 2/10C	6/10C *vs.* 2/11	5	0*	0*	7	0†	0†	12	0†	0†
3. 4/7 *vs.* 13/15	4/15 *vs.* 7/13	4/13 *vs.* 7/15	5	0*	0*	4	1	1	9	1*	1*
4. Map (M) tree	Sequence (S) tree		4	0		0	1		4	1	
5. M or S tree	2 next to 14/12/15 or 14		6	0*		6	1		13	2†	
6. M or S tree	3/4 next to 11/13 or 10–13		6	0*		8	1*		15	2†	
Placement of *P. halli* (types 23C and 23J)											
1. 1/23C *vs.* 17G/21	1/21 *vs.* 17/23	1/17 *vs.* 21/23	3	1	1	8	3	1*	11	4	2*
2. 8/23J *vs.* 16/18	8/18 *vs.* 16/23	8/16 *vs.* 18/23	4	0	1	8	3	2	12	3*	3*
3. 10C/23C *vs.* 17F/19	10/19 *vs.* 17/23	10/17 *vs.* 19/23	4	1	2	10	1†	2*	14	2†	4*
4. Map (M) tree	Sequence (S) tree		3	0		2	9*		5	9	
5. M or S tree	23C/23J next to 12/15		3	1		5	1		7	2	
6. M or S tree	23J next to 16/17		3	0		7	0†		9	2*	

In tests 1–3 in each set, the four-taxon networks are described by two pairs of numbered mtDNAs (see Table 6 and Figure 8) joined by a central branch ("*vs.*"). A slash indicates that the taxa are meant to form a monophyletic group. Map trees are those in Figures 6A (intra-*P. temporalis* comparisons) and 7A (tests of placement of *P. halli*), while the sequence trees are those in Figs. 6B and 7B for the same comparisons. In tests 5 and 6, for tests using map data, the map tree was tested against the indicated alternative, while in tests using sequence data, the sequence tree was tested against the indicated alternative. Also, in tests 5 and 6 in each set, when the combined data were used, the data were made to conform to the appropriate sequence tree (Figures 6B or 7B), then tested against the indicated alternative; thus, the results of tests using the combined data are not necessarily sums of the tests on map and sequence data. The probability, computed with a one-tailed binomial test, that trees A and B or A and C are statistically equally parsimonious in accounting for the observed distribution of sites is indicated with symbols next to the number of sites favoring trees B and C as follows: *, $P < 0.05$; †, <0.01. The number of steps a given site underwent on trees that possess polytomies was determined by the "soft polytomy" method of MADDISON (1989); in this method the minimum number of steps of each site on a polytomous tree is determined by finding the minimum number of steps of each site on fully resolved trees that are compatible with the polytomous one.